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ON THE PROBLEM OF THE INVASIVENESS OF THE PLAGUE BACILLUS

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ON THE PROBLEM OF THE INVASIVENESS OF THE PLAGUE BACILLUS

- USSR -

/Following is the translation of an article by I. V. Domaradskiy in the Russian-language periodical Izvestiya Irkutskogo Gosudarstvennogo Nauchno-Issledovatel'skogo Protivochumnogo Instituta Sibiri Dal'nego Vostoka (Journal of the State Scientific-Research Anti-Plague Institute of Siberia and the Far East, Irkutsk), Vol 21, 1959, pages 108-121./

Virulent microbes have various abilities for penetrating into the organism, or various degrees of invasiveness. At the present time it has been established that the invasive ability of microbes is connected with the changes in penetrability of the connective tissue brought about by the microbes.

Connective tissue consists of cellular elements (fibroplasts, macrophages, fat cells), fibrous elements (collagenous, reticular and elastic fibers) and interfibrillar substance, the "basic substance" (mucopolysacchrides, protein components), (Regan, 1953).

The basic substance of the connective tissue, which apparently is an optically homogeneous colloid in the form of an elastic gel, is very important in relation to invasiveness. It must be noted, that as in the case of all gels the basic substance has an internal structure lacking division of the dispersed phase and the dispersion medium. This also explains the absence of interstitial or tissue fluids in the connective tissue under normal conditions. According to the data of Shade and Menshel' (1923) only a few drops of fluid may be squeezed from subcutaneous connective tissue, even at fairly high pressure. It may be mentioned also, that the viewpoint asserting the existence of spaces ("fissures") in the connective tissue, containing free fluid, was disproved by the results of the investigations of Gyul'ze (1918) and Guek (1920).

The dispersion phase of the gel of the basic substance is a plycoproteid. It is stained metachromatically by toluidine blue, is digested by trypsin (but not by pepsin) and is precipitated by alcohol, acetone, trichloracetic acid, mercuric chloride and other reagents.

During recent years Day (1948, 1949, 1952) investigated the submicroscopic structure and physico-chemical properties of the basic substance. The present author undertook clarification of the relationship of the basic substance of connective tissue to visible structure elements of mesenchymal tissue, to collagenci and to reticular fibers. After processing with alcohol, Day discovered a very fine fibrillar structure in the tissue fissures, which to that time had been believed to be empty. These fine fibrils, visible only under the highest magnification, are not identical with connective tissue fibers, and become visible only upon treatment with alcohol. Upon hydration of the connective tissue the basic substance between the fibers, and not the collagenic fibers, themselves, swells or thickens.

Day indicated further (1952), that the amount of fluid filtering through a thin fascia membrane per unit time may be increased 10- to 20-fold by the addition of hyaluronidase to the fluid or through treating the fascia with it. The effect of hyaluronidase was reduced with the addition of starch to the fluid.

If the permeability of a membrane has been normalized by starch to the extent that filtration cannot be increased with hyaluronidase, filtration may be increased with ptyalin. Filtration also may be slowed down by solutions of dextran of various molecular weight (97,000 to 400,000). The effect of dextran depends both upon the concentration, and upon the size of the molecule.

On the basis of his investigative results Day came to the conclusion that the polysaccharides decomposed by hyaluronidase are located in loops of the protein network. The protein structure of the gel of the basic substance thus acts as a mesh, the openings of which are plugged by mucopolysaccharides under normal conditions. The polysaccharides are split by the hyaluronidase, and in this case they may be replaced by other macromolecules (i.e., permeability is increased).

Day also substantiated these results through the use of the electronic microscope. From the photographs it is clearly seen that the macroscopic astructural basic substance is formed of numerous fine fibrils. This type of structure is characteristic of a homogeneous substance. Preliminary processing with hyaluronidase has no effect on the structural elements. This indicates that polysaccharides are not included in their composition (Day and Eaves, 1953).

It must be emphasized that acidic mucopolysaccharides may comprise a substrate for the depolymerizing function of hyaluronidase, but muco- or glycoproteids may never serve in this role. The last named

may serve as a substrate for beta-glucosaminidase of extracts of testicle (semennika), just as the mucoproteid hormones of the anterior portion of the hypophysis and of gonadotropic hormone of the chorion serve as substrates for mucinase of the grippe virus and of Vibrio cholerae (Whitten, 1948).

As a result of extensive study of mucopolysaccharide components of mesenchymal tissue Meyer and Rappoport (1951) divided these mucopolysaccharides into five types: hyaluronic acid, hyaluronosulphate, and three types of chondroitin sulphate: A, B and C. Hyaluronosulphate, which is found only in the cornea, is not of particular interest as a substrate for hyaluronidase despite the fact that it is rapidly depolymerized by testicular and pneumococci hyaluronidases. However, other types of mucopolysaccharides are widely distributed. Hyaluronic acid has been discovered in sinovial fluid, in the skin and in the umbilical cord. Chondroitin sulphate A has been identified only in the hyalin cartiledge, chondroitin sulphate B in the heart valves, aorta and tendons, and chondroitin sulphate C in all the aforementioned, with the exception of the sinovial fluid.

At the time Chain and Duthie (1940) completed their research hyaluronidase was attributed an important role in increasing the permeability of tissues. In particular, it was proven that hyaluronidase accelerates the migration of both electrolytes (Forbes et al., 1950) and proteins (Banks et al., 1949) from subcutaneous cells to the blood. However, they attributed special importance to hyaluronidase in the development of diseases caused by pathogenic micro-organisms. Hyaluronidase was discovered in many types of bacteria, including streptococci (Duran-Reynals, 1933, Crowley, 1944), staphilococci (Duran-Reynals, 1933), pneumococci (Mas-Clean, 1936; Meyer, Dubos and Smyth, 1937), the causative agents of anaerobic infections (Meyer, Hobby, Chaffee and Dawson, 1940, Mac-Clean, Rogers and Williams, 1943) and diphtheria microbes (Mac-Clean, 1941).

Javits and Meyer (1943) found through application of the method employed by Menkin that extracts of avirulent and virulent strains of plague microbes contained a factor which increased the diffusion of the microbe and the permeability of capillaries. These authors, however, did not undertake special study of the Duran-Reynals factor, and treated this problem merely incidentally. Due to this fact the results of the investigations of Korobkova (1947, 1950, 1951, a and b) are of especially great interest.

The following preparations were used by Korobkova (1947) in isolation of the Duran-Reynals factor in plague microbes:

(1) a suspension of live virulent and avirulent plague microbes;

(2) lysates prepared by means of washing dense agar cultures with distilled water, followed by 20-fold chilling, thawing and heating at 58 degrees Centigrade for one hour;

(3) encapsulated antigen;

(4) centrifugal removal of transparent fluid from a wash of a two-day, thick agar culture;

(5) filtrates of this fluid.

Korobkova indicates that a more or less active factor was discovered in all preparations of plague microbes which increases the normal permeability of the tissue, and the most active factor was that obtained by centrifuging from a wash of agar culture fluid. The permeability of the skin increased also in cases in which the preparations were introduced directly into the vascular system.

It was established also, that guinea pigs immunized with live plague vaccines become resistant to the plague microbe diffusion factor; with intradermal introduction of plague microbe extract in rabbits the status of local immunity to the diffusion factor is limited only by the area of preliminary treatment.

Korobkova later came to the conclusion (1950) that the ability of plague microbes to increase the area of dispersion of stains in the skin of the rabbit and guinea pig is determined by the presence in the animal of an enzyme, which she identified as hyaluronidase.

According to the data of Korobkova, hyaluronidase of the plague microbe is relatively thermostable. Heating active preparations at 58°C merely weakens them; heating at 100°C for one hour also fails to destroy this enzyme completely.

Anti-plague sera obtained from animals immunized with strains containing hyaluronidase neutralize the enzymatic action of the microbe. Antihyaluronidase neutralizes the enzyme of both homo-, and heterologous strains.

In consideration of the foregoing it appeared entirely indicated that we undertake investigation of the pathogenetic factors of the plague bacillus with the study of hyaluronidase. However, we soon became aware of a disagreement between our data and the results of the work of Korobkova (Domaradskiy, Yaromyuk and Vasil'yeva, 1958).

We determined the initial activity of hyaluronidase by the method of Smirnova. Unfortunately this method contains many shortcomings. One of these is the impossibility of utilizing centrifugates of thick suspensions of bacteria, as recommended by Korobkova. Addition of 0.2 ml of 2 N solution of acetic acid to the substrate-centrifugate suspension system containing $5 \cdot 10^{10}$ to 10^{11} microbe bodies per

milliliter resulted in the formation of no clots, even if the bacteria contained no hyaluronidase. Thus it is possible to arrive at incorrect conclusions unless a suitable number of control experiments are performed. Furthermore, evaluation of the results is fairly difficult in the case of application of bullion cultures of the microorganisms (cf. supplementary information by Predtechenskiy, 1950, and Taratorina, 1947). The method of Smirnova yields repreducible results only if diluted suspensions of bacteria ($2 \cdot 10^9$ microbe bodies per milliliter) are used.

For this reason, we used the method of Mac-Clean, in the Mogilevskiy and Kogan (1949) modification, which does not have the shortcomings indicated above, in a major portion of our investigation. Two variations of the method were used. First, we used aqueous extracts of fresh or acetone dried umbilical cord without subsequent precipitation of the hyaluronic acid with alcohol. Second, the viscosity of the aqueous extracts of hyaluronic acid was varied within a fairly wide range (from 2.3 to 6.2) /see Note/ in different series of experiments.

(/Note/ In our opinion substrates with different degrees of viscosity enable demonstration of hyaluronidases with various degrees of activity.)

Hyaluronidase was identified in 20 strains of Bact. pestis /see Note/, in one non-typified strain of streptococcus, and in Staphylococcus aureus and Bact. perfringens.

(/Note/ Avirulent strains YeV, 1, 17, 50/74, 154; virulent strains TsD, 94-96, 119, 125, 143, 435, 483, 485-488, 1435 and 1525).

The causative agent of gas gangrene was cultured on the Kitt-Tarozzi medium. Hottinger and Marten heat treated preparations served as the main medium for cultivation of the three other types of microorganisms.

Plague bacillus was cultivated on liquid and solid media, and strepto-, and staphyllococcus were cultivated only on solid media, in the presence of 5 percent blood solution. The period of cultivation was 24 to 48 hours on solid media, and 20 hours in liquid medium.

In using microorganisms in conjunction with agar medium the concentration of microbe bodies was $2 \cdot 10^9$ and $5 \cdot 10^{10}$ per milliliter. Suspensions of non-washed bacteria and the overlying liquid obtained upon centrifuging were used in the investigation. In the application of liquid media the hyaluronidase activity was determined mainly in non-filtered cultures.

Aqueous and saline extracts of defatted bull testicles were used as the preparation known to contain the given enzyme; the testicle material was partially freed of its accompanying matter through dialysis and processing with 0.1 N acetic acid.

The same objects of investigation, inactivated by heating above a boiling water bath for 10 minutes, were used as control experiments.

The results of the experiments are shown in Table 1.

TABLE 1. RESULTS OF DEMONSTRATION OF HYALURONIDASE IN EXPERIMENTS IN VITRO

Object of Investigation	Number of Experiments	Activity /see Note/
Plague bacillus	42	0
Streptococcus	8	42
Staphyllococcus	5	52
Perfringens	9	3,072
Testicular Extract	7	3,072

(/Note/ The unit of activity was taken as the greatest dilution of enzyme preventing formation of hyaluronic acid clots. The figures indicate the number of activity units of hyaluronidase per milliliter undiluted fluid.)

It is apparent from the table that the specimen strains of plague microbe are unable to depolymerize hyaluronic acid. In the other types of microorganisms the hyaluronidase is demonstrated under identical experimental conditions. The greatest activity was exhibited by the enzyme of bullion cultures of Bac. perfringens. The activity of our strain of causative agent of gas gangrene did not differ from that of the strains used by Ogloblina (1948). However, the activity of hyaluronidase in the case of strepto- and staphyllococcus varied considerably, which was in conformity with data on the adaptive nature of the enzyme as indicated in the technical literature (Rogers, 1941; Mac-Clean and Hale, 1941). At the time of conclusion of the present investigation, the streptococcus strain had lost the capacity to deplymerize hyaluronic acid following repeated transplantation onto synthetic nutrient media. The enzyme loss was a fairly stable characteristic. During the process of repeated transplantation of Staphyllococcus aurens onto media containing hyaluronic acid the activity of the enzyme increased twofold (with single dilution).

H aluronidase was not detected in plague bacillus in thick suspensions subjected to autolysis at 37°C for two to ten days.

Similar results were obtained with our experiments in vivo. The investigations were conducted upon rabbits with light skin, irrespective of species. The ability of the bacterial culture to increase

the permeability of tissue was tested by means of intradermal injection of 0.2 ml mixture of supernatant fluid or bullion culture filtrate, plus 0.75 percent solution of trypan blue in rabbits. The following injects were performed simultaneously for control purposes:

(1) dyes and physiological salt solution;

(2) dyes and inactivated preparation;

(3) dyes and sterile bullion.

The diffusion activity of the investigated objects was noted 24 hours after their injection.

A total of 15 experiments were performed, of which eight involved plague bacillus cultures (strains YeV, 17 and 154). The data of the experiments are shown in Table 2.

TABLE 2. RESULTS OF DEMONSTRATION OF THE DIFFUSION FACTOR IN EXPERIMENTS IN VIVO

Object of Investigation	Coefficient of Dye Diffusion		
	With Sterile Medium	With Heated Object of Investigation	Unheated Object of Investigation
Centrifugate of agar culture of Strain YeV of plague bacillus	1.8	1.6	1.7
Filtrate of bullion culture of perfringens	1.2	2.6	5.5
Testicular Extract	-	1.3	3.9

In all cases the area of diffusion of the dye was approximately equal (within the limits of accuracy of measurement) upon injection of the rabbits with either unheated or inactivated centrifugates of plague bacillus cultures. Usually the coefficients of diffusion for injection of centrifugates differed very little from the coefficients of diffusion of the sterile medium.

Pathological changes at the site of injection of the plague bacillus consisted of moderate diffusion, and usually perivascular, inflammatory infiltration of the subcutaneous cells by polymorphonuclear

leucocytes with an admixture of hystiocytes and lymphocytes. A similar picture is observed at the site of injection of Hottinger's bullion. Injection of a physiological salt solution evoked no visible inflammatory changes /see Note/.

(/Note/ We express our thanks to Candidate of Medical Science R. S. Kolesnik, who conducted the morphological investigations.)

What is the reason for the difference between our data and the results obtained by Korobkova? It is difficult to answer this question because Korobkova does not describe in detail the method she used in any of her works. We may merely presume that she used the Smirnova method in the determination of hyaluronidase in vitro.

Failing to obtain the expected results with $2 \cdot 10^9$ suspensions of bacteria, Korobkova used thicker mixtures (25-, to 50-fold thicker). In this she encountered the phenomenon of non-specific inhibition of coagulation of hyaluronate (upon addition of acetic acid), resembling the action of hyaluronidase /see Note/.

(/Note/ It must be noted that similar indications of the possibility of obtaining non-specific inhibition of hyaluronate coagulation may be found in the works of several authors. Two of these authors have been cited in the above. We may adduce another example at this point. At the Inter-Institute Conference on Scarlet Fever and Diphtheria held in Moscow in 1948, Lyampert (1950) stated: "Undiluted meat-peptone bullion and concentrated washes with agar medium usually give a non-specific reaction." Many speakers at this conference (Sadovskiy, Ioffe and others) brought up the question of the unification of the method of determination of the activity of hyaluronidase, in view of the fact that lack of such unification is the basis of frequent misunderstandings.

The second reason apparently is the fact that Korobkova failed to use control experiments for inactivated preparations and for the sterile medium in her experiments in vivo.

Furthermore, evaluation of the results of reactions in Korobkova's experiments was complicated by the fact that hyperemia and extensive infiltrates formed at the site of injection of plague preparations in almost all cases, with subsequent frequent formation of hemorrhagic necrosis.

In essence, the magnitude of the coefficients of diffusion were functions of the acuteness of the "skin affections." Actually, the reaction was stronger in the case of injection of the animal with cultures of virulent bacteria than upon injection with avirulent cultures; on the other hand, lysates evoked "a slight degree of skin

affection" in guinea pigs, as recorded by Korobkova (1947), regardless of whether they were prepared from virulent, or avirulent plague strains, and contained a less active diffusion factor.

This gives the impression that the degree of development of the inflammatory process served as a criterion for evaluation of the diffusion ability of the microbe in Korobkova's investigations.

It should be mentioned, also, that in her later articles devoted to the factor of diffusion of the plague bacillus Korobkova often drew conclusions which were based either on inadequate experimental results, or were based on external analogies and not supported by definite experimental materials. One example is the fact that in one of her works (1951) she writes: "In experiments in vitro it was noted that freshly prepared suspensions of plague bacillus, in distinct on from other preparations, did not reduce the viscosity of hyaluronate. Characteristic mucin clots were formed upon addition of acetic acid to the live microbe + hyaluronate system. In experiments with animals, however, these suspensions increased the diffusion of dye in the skin, similar to the in vitro reactions of preparations such as hyaluronidase. Investigation of this phenomenon led to the discovery of hyaluronic acid in the plague bacillus."

From these quotations it is apparent, first of all, that live plague bacilli do not contain hyaluronidase. Therefore, our data are substantiated.

Secondly, the increase in diffusion of dye in the skin occurred apparently as a result of the inflammatory reaction caused by the microbes.

Finally, an unexpected conclusion is drawn relative to the presence of hyaluronic acid in the plague bacillus. What is the basis for this conclusion? It is based on references to the work of Siston, quantitative tests with acetic acid evoking the formation of clots in microbe suspensions or in supernatant fluid, and the ability of testicular hyaluronidase to lower the viscosity of plague cultures.

However, postulating the simultaneous presence of hyaluronidase and the substrate of its action, hyaluronic acid, in the plague bacillus, Korobkova does not cite the work of Mac-Clean (1941, a and b), which indicates that the diffusion factor never is formed by encapsulated streptococcus, and that the latter may be decapsulated by hyaluronidases deriving from capsule-less streptococci or from other sources. Thus the capsule and hyaluronidase are mutually exclusive, because the enzyme either impedes the formation of the capsule or destroys the formed capsule. Nevertheless, Korobkova states (1951) that "the small amount of hyaluronidase, initially formed in the culture, is connected with the excess substrate (hyaluronic acid), which leads to an increase of the production of this enzyme by the bacterial cell."

In the opinion of Korobkova the ability of testicular hyaluronidase to reduce the viscosity of plague cultures serves as another indication of the presence of hyaluronic acid in this species of microorganism. However, as mentioned in the foregoing, hyaluronidases (especially that derived from the testicle) are not characterized by strict specificity, and together with hyaluronic acid, act on other polysaccharides.

The third indication of the presence of hyaluronic acid in plague bacillus based on the formation of clots in microbe suspensions by acetic acid need hardly be mentioned in the present article.

The data cited in the foregoing indicate that the problem of the hyaluronidase of plague bacillus may not be considered resolved. However, the fact of its rapid diffusion in the infected organism is beyond doubt. This fact should be studied in subsequent investigations. Determination of the ability of bacterial strains to form hyaluronidase in media not containing hyaluronic acid may produce negative results despite the fact that these strains do produce hyaluronidase in media containing hyaluronic acid, or in vivo. It has been proven that strains of certain bacteria which are able to form hyaluronidase in the presence of specific substrate utilize several hyaluronic acid decomposition products as inducers, especially N-acetylglucosamine, which for practical purposes is not secreted and is not decomposed by the macroorganism (Mac-Clean and Hale, 1941). Amino acids and peptides, as well as hyaluronic acid and products of its decomposition, may influence the synthesis of hyaluronidase by microbes. This is substantiated by the investigations of Mergenhagenn (1958), proving that Staphyllococcus aurens AB 2 synthesizes hyaluronidase on a medium of given chemical composition only in the presence of tyrosine and tryptophane, although these amino acids are not necessary for growth. Glycyl-L-tyrosine and glycyl-L-tryptophane have similar effects. However, the substances which induce the formation of hyaluronidase by one microorganism do not necessarily effect another microbe (Rogers, 1946).

It must be mentioned also, that the effect of hyaluronidase on hyaluronic acid may be broken down into two clearly distinguishable processes: depolymerization, which occurs rapidly, and the slower process of hydrolysis, which liberates acetylglucosamine and glucuronic acid. This indicates the presence of two enzymes, which effect a splitting of various glucoside bonds in hyaluronic acid (Meyer, 1947; Hahn, 1945, a and b, 1946, a and b; Rogers, 1946, a and b, and others). The existance of two different enzymes is supported by observations according to which the pneumococcic hyaluronidase hydrolizes the substrate almost completely, although testicular hyaluronidase catalyzes the hydrolysis only 50 percent during this period, reducing the viscosity of the substrate solution somewhat more rapidly than does the bacterial enzyme. Some data indicate that different products result from the hydrolysis of hyaluronic acid by bacterial hyaluronidase and by the same enzymes derived from testicle material.

In addition, the activity of the enzyme decreases as a function of the pH of the medium, the concentration of electrolytes and colloids in the medium, the temperature and many other factors. (Mac-Clean, 1943; Rogers, 1948; Meyer et al., 1941; Madinaveitia and Quibell, 1941; Mogilevskiy and Kogan, 1949.)

From the foregoing it follows that the results obtained in determining the activity of hyaluronidase derived from various sources or in measuring the activity of one and the same hyaluronidase by different methods frequently may give varying values.

If in the future our data are substantiated and it is found that the plague bacillus belongs to the group of hyaluronidase-negative bacteria, does this reflect substantially on our propositions concerning the pathogenesis of plague? Obviously the answer is negative.

Many authors (such as Murray, 1955) deny the fact of complete correspondence between the invasiveness of a bacillus and its ability to produce hyaluronidase. Some bacteria having high invasiveness do not form this enzyme. This may be observed particularly in several strains of perfringens (Eavens, 1943) and streptococci (Crowley, 1944). On the other hand Staphyllococcus auriens, a microbe with relatively low invasiveness, forms large amounts of hyaluronidase. In these cases, however, when the microbe forms hyaluronidase it must not be thought that it may diffuse without limit in the organism.

It is known that the blood contains no substances having hyaluronidase action. On the contrary, hyaluronidase introduced into the blood stream is quickly inactivated. Under normal conditions the antihyaluronidase titre of the serum is a fairly constant index, although it may increase during several diseases (not necessarily infectious diseases). For example, the antihyaluronidase titre increases during rheumatism (Friou and Wenner, 1947; Quinn, 1948, Harris and associates, 1949), in poliomyelitis (Glick and Gollan, 1948), in pneumonia (Thompson, 1948), glomerulonephritis (Harris et al., 1950) and in shock (Cole, Shaw and Frazer, 1950). According to the data of Lyampert, Halperin and Ralph (1950) an increase in the titre of substances neutralizing hyaluronidase obtained from hemolytic streptococcus Type 4 is noted in scarlet fever patients, irrespective of the type of streptococcus isolated from these patients. It is of special interest that the increase in antihyaluronidase titre also may be noted in patients excreting various types of hemolytic streptocci which do not produce hyaluronidase.

The problem of the nature of the substances causing antihyaluronidase action in vivo has not been definitely resolved. It is more correct to say that the actual substance which plays the decisive role is difficult to identify.

Haas (1946) was the first to discover an enzyme in normal blood plasma which destroys hyaluronidase. He named this substance anti-invasin-I. This enzyme has been discovered in the plasma of all tested species of mammals, birds and fish. Antiinvasin-I acts on various hyaluronidases, regardless of their source of origin.

Haas later discovered the new enzyme proinvasin-I on media on which several pathogenic bacteria were growing, and in snake venum. This enzyme is formed by microbes which form hyaluronidase, and its function is related to the destruction of antiinvasin-I. The blood of animals also contains an enzyme acting upon proinvasin-I (antiinvasin-2).

Detailed investigation of the reaction between serum inhibitor and hyaluronidase revealed that the indicated reaction occurs very rapidly, and that the speed of this reaction accelerates with a decrease in temperature. This fact contradicts the proposed enzymatic nature of the inhibitor.

The serum inhibitor was isolated in pure form by Mathews and Dorfman (1955). It was found to be a considerably thermolabile antibody (Dorfman, 1950; Waltenberg and Glick, 1952). It was proposed that the serum suppressor is a heparin-protein complex (Glick and Silven, 1951). The significance of heparin in inhibiting the activity of hyaluronidase was indicated by Bagdi and his associates (1950). The mechanism of inhibition apparently is based on the fact that this enzyme combines with heparin, the structure of which is fairly similar to that of hyaluronic acid. However, no direct indication of the presence of heparin in purified preparations of the serum inhibitor were discovered (Waltenberg and Glick, 1952).

Mac-Clean (1943) reported that immunization of rabbits with hyaluronidase obtained from bull testicle material, from perfringens cultures, from staphyllococci and from hemolytic streptococci leads to the formation of antibodies which neutralize the action of hyaluronidase. In the experiments of Mac-Clean the immune sera were neutralized only with the hyaluronidase which produced the immunity. According to the data of Hoggy et al. (1941) the antihyaluronidase of streptococcus and pneumococcus inhibit the action of this enzyme in vitro, but do not affect the activity of their factors of diffusion in the skin. They proposed that the enzyme-antienzyme complex dissociates readily in vivo.

However, the data cited above, and the data obtained from clinical material, which is not discussed in the present article, are inadequate for testing the validity of the antigenic properties of hyaluronidase. Proof of antigenic properties of this, or any other enzyme, requires the use of pure preparations. Only then may it be demonstrated that the antibody is formed in response to the introduction of the enzyme, and

not in response to the introduction of substances accompanying it. Apparently the formation of antibodies in relation to the substances accompanying the hyaluronidase was caused by the specificity of the action of the corresponding sera in the experiments of Mac-Clean and the contradiction in the results of the investigations of Hoggy and his associates.

Unfortunately we are unable to discuss the problem in question in greater detail, and refer the reader to the original work of Bakh (Bakh, 1950).

To avoid misunderstanding we repeat that the foregoing is intended merely to indicate the difficulty of resolution of the problem of the mechanism of the antihyaluronidase action of the blood.

If there is not complete correspondence between the invasiveness of a microbe and its ability to produce hyaluronidase, what enables the diffusion of hyaluronidase-negative microbes in the organism, especially those such as pasterellosis, brucellosis, some types of salmonellosis and rickettsia viruses?

First of all, it has been proposed that the factors of diffusion are substances of extremely diverse nature. Many of them have no enzymatic action upon hyaluronic acid (Duran-Reynals, 1942). These include, for example, collagenase, which forms various types of clostridia. This enzyme, which destroys the collagenci fiber, increases the permeability of the connective tissue (Gersh and Catchpole, 1949). More rapid dissemination of microbes also may be made possible by the existence of several of their abilities to dissolve fibrin.

Secondly, in many cases an increase in the permeability of the tissue is caused not by microorganisms, but by those changes which develop in the organism of the host in response to the introduction of a pathogenic agent. The latter circumstance must be taken into consideration in cases in which the given microorganism forms hyaluronidase. We may take the liberty of asserting that this so-called "nonspecific" factor of increased permeability plays a fairly important role in the diffusion of microbes in the organism.

All the factors which increase distribution of the microbes may be divided into the following two groups:

(1) those acting predominantly upon the basic substance of the connective tissue, particularly the mucopolysaccharides, and

(2) those which change the permeability of blood capillaries.

The above division is purely arbitrary. It is impossible to indicate precisely where the effect on the connective tissue ends and change in the permeability of capillaries begins. Hyaluronidase, the main

function of which is depolymerization of hyaluronic acid, influences the permeability of capillaries (Benditt et al., 1951). In general, everything which increases the permeability of capillaries also enables an increase in the speed of diffusion in the connective tissue, and conversely, changes in the functional status of the connective tissue, especially in its structure, are reflected in the permeability of the capillaries (these problems are discussed in greater detail in the monograph of Rusznyak, Foldi and Szabo, 1957).

It has been established by numerous investigations that the processes of distribution of various agents (crystalloids and colloids) in the organism is regulated by endocrinal and nervous factors.

In 1940 Weinstein indicated that extracts of the anterior lobe of the hypophysis reduce the distribution in the connective tissue; a similar effect is produced by extracts of the posterior lobe of the hypophysis (Favilli, 1939) and of the adrenal cortex (Menkin, 1940). Reduction of the permeability of the connective tissue and inhibition of the activity of hyaluronidase in vivo also may be obtained through the introduction of adrenocortical hormone (Vogt, 1944; Long and Fry, 1945; Lurie, 1950), or through another form of stimulation of the function of the adrenal cortex; morphine, subcutaneous injection of formalin, high temperature, or cold (Favilli, 1939; Opsahl, 1949, a; Cahen and Grainer, 1944; Shiman and Finestone, 1950; Birke, 1953). On the other hand, adrenalectomy leads to an increase in the permeability of the connective tissue (Opsahl, 1949, b).

Metabolic poisons, especially moniodoacetic acid, arsenate, cyanide, fluroides, etc., also have great effect upon the permeability of the connective tissue.

The reagents mentioned above act not only upon the capillary walls, but also upon the basic substance of the connective tissue.

Not long ago it was considered that the nervous system has no direct effect upon the connective tissue because there is no direct innervation of its structural elements, the fibers and the basic substance. However, very recently Kiss and Lang (1954) indicated that the collagenic fibers of the connective tissue are connected with the vegetative nervous system. According to their data characteristic nervous plexi exist at the surface of the collagenic fibers (the iris, gall bladder, circular ligament of the hip, etc.) and also within them.

The facts discussed in the foregoing emphasize the complexity of the problem of the dissemination of microbes in the organism, and the fact that resolution of this problem may be attained only through clarification of the hyaluronidase of microbes.

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